

# Tremorgenic Toxin from *Penicillium verruculosum*

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Received for publication 7 April 1972

A new mycotoxin that produces severe tremors and acute toxicity when administered orally or intraperitoneally (ip) to mice and 1-day-old cockerels was obtained from a strain of *Penicillium verruculosum* Peyronel isolated from peanuts. The ip 50% lethal dose (LD<sub>50</sub>) of this tremorgen was 2.4 mg/kg in mice and 15.2 mg/kg in chickens. Orally administered LD<sub>50</sub> values for the toxin were 126.7 mg/kg in mice and 365.5 mg/kg in chickens. The trivial name "verruculogen" is proposed for this tremorgenic mycotoxin. Physical and chemical characteristics of the mycotoxin are described.

The production by fungi of secondary metabolites that elicit a severe tremorgenic response when administered to animals has been reported (1, 2, 4, 5). Wilson and Wilson (4) isolated a tremorgenic substance from *Aspergillus flavus*. The toxin affected mice, rats, and guinea pigs whether administered orally or intraperitoneally (ip). In addition to tremors and convulsions, the tremorgen caused acute toxicity in mice. Three isolates of *Penicillium cyclopium* produced a chemical that evoked neurotoxic symptoms similar to those caused by the compound from *A. flavus* (5). *P. palitans*, a mold closely related to *P. cyclopium*, produced a tremorgenic mycotoxin which, when administered to mice ip, caused tremors with as little as 125 µg/kg (1). Thin-layer chromatography (TLC) of the *P. palitans* tremorgen with the *P. cyclopium* tremorgen and comparison of their ultraviolet (UV) spectra indicated they were identical (1). Recently Hou et al. (2) reported the production of the same tremorgen and two other similar, biologically active compounds by *P. palitans*, *P. cyclopium*, *P. crustosum*, and *P. puberulum*. The molecular formulae for two of these tremorgens were C<sub>37</sub>H<sub>44</sub>NO<sub>6</sub>Cl and C<sub>37</sub>H<sub>44</sub>NO<sub>6</sub>.

Yamazaki et al. (6) reported isolation and preliminary characterization of two tremorgens, fumitremorgin A and B, from *A. fumigatus*. Chemical composition of these tremorgens was reported as C<sub>33</sub>H<sub>45</sub>O<sub>6</sub>N<sub>3</sub> and C<sub>26</sub>H<sub>22</sub>O<sub>6</sub>N<sub>3</sub>, respectively. Administration ip of 1 mg each of pure toxins per mouse caused

sustained trembling with intermittent convulsions, but no acute toxicity was observed at this dosage level. Administration of 5 mg/mouse resulted in death of 70% of animals within 96 hr.

The isolation of a new tremorgenic mycotoxin produced by a strain of *P. verruculosum* obtained from peanuts is reported.

## MATERIALS AND METHODS

**Production and isolation of toxin.** The fungus identified as *P. verruculosum* Peyronel was cultured in Fernbach flasks (2.8 liter) containing 100 g of shredded wheat and 200 ml of Difco mycological broth (pH 4.8) supplemented with 1.6% yeast extract. After 14 days of growth at 28 C, cultures were extracted by blending three times with a total of 1 liter of hot chloroform. Chloroform extracts were pooled, filtered through cheesecloth, and then passed through anhydrous sodium sulfate in a Buchner funnel to remove spores and water. The extract was evaporated to dryness in vacuo, taken up in chloroform, and chromatographed on a silica gel (Merck 70-325 mesh) column (3.5 by 40 cm) packed in *n*-hexane. First, the oil was eluted from the column with 10 column volumes of *n*-hexane; the toxin was then eluted with 10 to 12 column volumes of ethyl ether. The toxin was purified further on a second silica gel column packed in toluene. The column was eluted with 1 liter of toluene, placed on an automatic fraction collector, and eluted with a linear gradient from toluene to ethyl acetate. Fractions (240) of 27 ml each were collected, and the tremorgenic activity was associated with the contents of tubes 114 to 170. Solutions in these tubes were combined and concentrated in vacuo, and the tremorgen

was further purified by using a chromatographic column packed with "Florisil" and eluted with 5% ethyl acetate in benzene. Tremorgenic activity was in tubes 63 through 80, inclusive. Active fractions were combined, concentrated in vacuo, and crystallized from benzene-ethyl alcohol solution (1:1, v/v).

The toxin was chromatographed by TLC (0.25-mm MN-Kieselgel G-HR), and the plates were developed in either chloroform-acetone (93:7, v/v) or toluene-ethyl acetate-formic acid (5:4:1, v/v/v). The toxin produced spots visible in normal light or long-wave UV light after developed plates had been sprayed with 50% ethanolic sulfuric acid.

**Biological assay.** Female Swiss mice (20 g average) and 1-day-old DeKalb cockerels (38 g average) were used for bioassay. The toxin was administered to the animals either orally or ip in corn oil. Toxin was prepared for dosing in the following manner. Toxin was dissolved in chloroform, the desired amount of corn oil was added to the chloroform solution, and chloroform was subsequently removed under vacuum at 70 C to form what appeared to be a solution of the toxin in corn oil at the levels tested. Chloroform in corn oil blanks treated in the same manner served as controls to insure removal of all chloroform prior to dosing. The oral 1-day-old cockerel bioassay was used to monitor the initial purification of the tremorgenic toxin.

**Physical and chemical analyses.** Infrared (IR) spectra were taken with a Perkin-Elmer model 257 IR spectrophotometer equipped with a 4X beam condenser. Samples were coated onto KBr blocks as a thin film. UV spectra of the toxin were taken with a Beckman model DB-G recording spectrophotometer in 95% ethanol solution. Mass spectral analyses were made with an A.E.I. Ms-9 mass spectrometer. Samples were introduced into the instrument by the direct probe method and ionization was effected by electron-impact at 70 eV or chemical ionization with isobutane as the ionizing reagent. Melting points were taken with a Kofler micro-melting point apparatus, and tests for the presence of chlorine were made by elemental analysis and isotope ratios in the same spectral analysis.

## RESULTS AND DISCUSSION

The trivial name "verruculogen" is proposed for this mycotoxin.

**Physical and chemical properties of the toxin.** The purified toxin appeared as a single spot on TLC plates developed with either chloroform-acetone (93:7, v/v,  $R_F$  0.30) or toluene-ethyl acetate-formic acid (5:4:1, v/v/v,  $R_F$  0.65). The toxin was visible immediately after spraying with 50% ethanolic sulfuric acid as a dark slate-gray spot in light or as a bright mustard-colored fluorescent spot under long-wave UV light. The toxin was soluble in benzene, ethyl acetate, and acetone, slightly soluble in ethanol, and very soluble in chloroform. Crystallization from benzene-ethyl alcohol solution yielded colorless crystals (plates,

melting point 233 to 235 C,  $[\alpha]_D = -27.7^\circ$ ). Approximately 2.1 g of verruculogen was recovered from 6.75 kg of culture medium (311 mg/kg).

UV analysis of verruculogen showed  $\lambda$  max ETOH 224 ( $E = 47,900$ ), 268 ( $E = 8,760$ ), and 294 nm ( $E = 9,710$ ), typical of an indole alkaloid system. The IR spectrum showed absorption maxima at 3,520 and 3,460  $\text{cm}^{-1}$  (OH or indole, or both), at 1,655  $\text{cm}^{-1}$  (carbonyl may be due to amide), and a doublet at 1,355 and 1,365  $\text{cm}^{-1}$  (possible *gem*-dimethyl). Low-resolution mass spectral analysis showed the heaviest detectable ion at nominal mass 511 m/e via electron-impact ionization. The heaviest detectable ion via chemical ionization was at nominal mass 551 m/e. Elemental analysis indicated a molecular formula of approximately  $\text{C}_{30}\text{H}_{37}\text{N}_3\text{O}_7$  (found: C, 64.39%; H, 6.52%; N, 7.44%). Tests for the presence of chlorine were negative. Studies are continuing to determine the structure of this toxin.

The toxin differs from the tremorgenic toxins reported by Wilson et al. and Hou et al. (1, 2, 4, 5) by the following characteristics: (i) its elemental composition, especially nitrogen (3 atoms/molecule compared to 1); (ii) its color reaction with  $\text{FeCl}_3$  in butanol (mustard colored vs. blue); (iii) its fluorescence under UV light after spraying with sulfuric acid and heating at 110 C (bright mustard compared to no fluorescence); (iv) and its melting point (233 to 235 C compared to 210 to 230 C for tremorin A and 185 to 195 C for tremorin B).

The toxin bears a closer resemblance to the tremorgens, fumitremogin A and B (6). Details on the chemical similarities and differences of verruculogen with the fumitremogins and a partial structure will be reported in a subsequent communication.

**Biological properties.** Verruculogen administered orally or ip caused severe tremors (Table 1) and acute toxicity in mice and cockerels (Table 2). Tremors began within 5 min after oral dosing and, depending on dosage level, persisted for several hours. Mice receiving lethal doses of tremorgen usually died during a tetanic convulsion within 45 min after treatment. Gross examination at autopsy revealed no apparent mode of action of the tremorgen.

Mice and cockerels showed similar threshold response (tremors) to the toxin when it was administered ip (Table 1). In acute toxicity tests, mice were six times as sensitive as cockerels to the toxin administered ip and three times as sensitive to toxin administered orally (Table 2).

TABLE 1. Lower limits of tremor response to verruculogen

Animal	Route	Dosage (mg/kg)	No. responding/no. treated
Swiss mice	ip	0.78	4/4
Swiss mice	ip	0.39	2/4
Cockerels	ip	0.66	5/5
Cockerels	ip	0.33	3/5
Swiss mice	Oral	40.00	5/5
Swiss mice	Oral	20.00	0/5
Cockerels	Oral	19.73	5/5
Cockerels	Oral	13.15	3/5
Cockerels	Oral	9.86	0/5

TABLE 2. Acute toxicity of verruculogen on mice and cockerels

Animal	Route	LD <sub>50</sub> (mg/kg) <sup>a</sup>
Swiss mice	ip	2.4
Cockerels	ip	15.2
Swiss mice	Oral	126.7
Cockerels	Oral	365.5

<sup>a</sup> LD<sub>50</sub> values were calculated by the formula of Weil (3).

The LD<sub>50</sub> of verruculogen (2.4 mg/kg, Table 2) is comparable to that reported for tremortin A (1.35 mg/kg) and tremortin B (between 2.76 and 5.52 mg/kg) when injected ip in mice (2). The lower dosage limit for tremor production in mice for the tremortins was reported as follows: 1.3 mg of tremortin B per kg injected ip caused tremors for several hours (2), and 0.125 mg of tremortin A per kg caused tremors that persisted for about 1 hr when injected in propylene glycol solution (1). The lower limit for tremor production by verruculogen injected ip was 0.39 mg/kg in Swiss mice and 0.33 mg/kg in cockerels (Table 1). The ip administration

of verruculogen was 40 times as effective in producing tremors in mice as orally administered tremorgen and 75 times as effective in cockerels (Table 1).

Clinical signs in mice and cockerels, in addition to tremors and acute toxicity, were hypersensitivity to sound, tetanic spasms, and ataxia. Signs more peculiar to cockerels were recumbency with the head extended forward and contracture of the leg tendons resulting in closure of the digits. Mice frequently were observed to rise on the hind legs and fall backwards. All clinical signs in mice and cockerels were exaggerated by enforced movement or fright. Animals receiving a sublethal dose of verruculogen appeared to be fully recovered between 24 to 48 hr after treatment.

#### ACKNOWLEDGMENTS

We thank Elmer E. Davis, American Type Culture Collection, for identification of the fungus and Malcolm J. Thompson, Insect Physiology Laboratory, USDA, Beltsville, Md., for performing the optical rotation analysis.

This investigation was supported by Public Health Service Research Grant FD-00081 from the Food and Drug Administration.

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